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HUMAN HEALTH SAFETY EVALUATION OF HALON REPLACEMENT CANDIDATES

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FOR THE DIRECTOR

David R. Mattie

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Environmental concern over the depletion of stratospheric ozone and global warming has led to an international treaty called The Montreal Protocol which calls for the phase out of halons by the year 2000. The services within the Department of Defense (DoD) are directed to determine and evaluate suitable halon replacement candidates that will optimize performance of mission-essential equipment and operations. Part of the evaluation process is to select halon replacement candidates that will be in compliance with environment, health, and safety considerations. In this document, a strategy for human health safety evaluation of halon replacement candidates is provided. A step-wise approach in building a chemical toxicity database, specific for halon replacement candidates, allows decisions to be made with budget and time constraints in mind. Four phases of toxicity testing are described. The confidence in predicting human health hazard increases as one proceeds from one phase of testing to the next, but the cost of each phase increases as well. Information is provided at the end of each phase that allows one to evaluate the overall benefit and cost of the tests performed before deciding to continue or stop toxicity testing of the replacement candidate. This research is part of the Department of Defense's Next-Generation Fire Suppression Technology Program (NGP), funded by the DoD Strategic Environmental Research and Development Program (SERDP). This document is part of the final reporting process for NGP Project 3B/1/89.

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PREFACE

This research is part of the Department of Defense's Next-Generation Fire Suppression Technology Program (NGP), funded by the DoD Strategic Environmental Research and Development Program (SERDP). This document is part of the final reporting process for NGP Project 3B/1/89. The research described in this report began in March, 1998 under Department of the Air Force Contract No. F41624-96-C-9010. Lt Col Stephen Channel served as the Contracting Officer's Representative for the U.S. Air Force, Air Force Research Laboratory/Operational Toxicology Branch (AFRL/HEST). Darol E. Dodd, Ph.D., served as Program Manager for ManTech Geo-Centers Joint Venture.

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HUMAN HEALTH SAFETY EVALUATION OF HALON REPLACEMENT CANDIDATES

INTRODUCTION

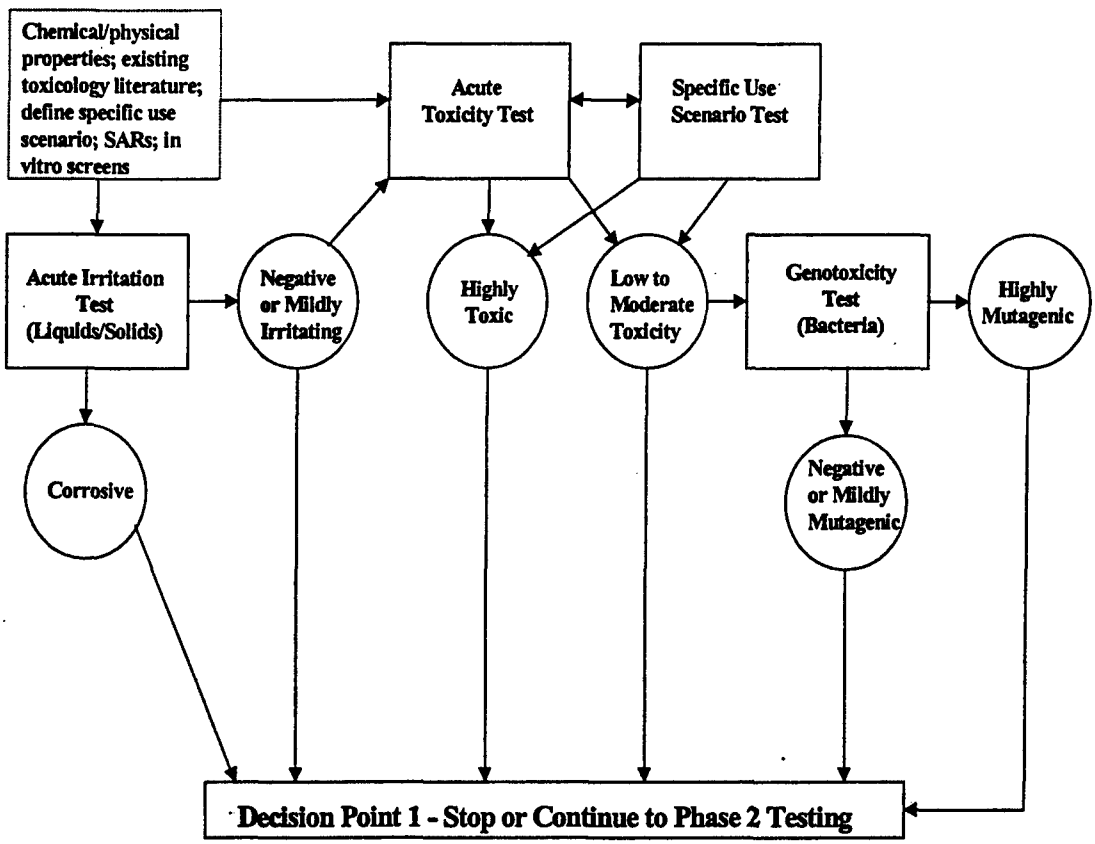
Environmental concern over the depletion of stratospheric ozone and global warming has led to an international treaty called The Montreal Protocol which calls for the phase out of halons by the year 2000. The services within the Department of Defense (DoD) are directed to determine and evaluate suitable halon replacement candidates that will optimize performance of mission-essential equipment and operations. Part of the evaluation process is to select halon replacement candidates that will be in compliance with environment, health, and safety considerations. The U.S. Environmental Protection Agency (EPA), under the Clean Air Act, is also directed to evaluate and regulate substitutes for ozone-depleting chemicals that are being phased out (Final Rule, Federal Register 59: 13044, March 18, 1994). The EPA program that provides these determinations is called the Significant New Alternatives Policy (SNAP) program. DoD and EPA share a common objective in identifying halon replacement candidates, that being, to select substitutes that offer low (or lower) overall risks to human health and the environment.

The purpose of this paper is to provide a strategy for human health safety evaluation of halon replacement candidates. This is necessary, because there are budget and time constraints associated with the identification and development of halon replacements. Decisions have to be made to meet project or program goals, yet information is not readily available to assist in making decisions on the toxicity evaluation of potential chemical substitutes. This paper will provide a stepwise approach in building a chemical toxicity database, specific for halon replacement candidates. Information will be provided on the types of toxicity tests or studies to be considered, their strengths, weaknesses, costs, and evaluation time. Suggestions will be provided when more than one choice arises and a decision has to be made. It is unlikely that a chemical substitute will require completion of all parts of the toxicity evaluation process. Once the evaluation process is stopped, information will be given on how valuable the database is in terms of uncertainty and risk to human health. The strategy described is based on the experience and opinions of the authors. Consideration of our comments and recommendations does not assure that no additional toxicity testing or research is needed on a halon replacement candidate to provide a complete assessment of the overall risks to human health.

PHASE 1 – TOXICITY SCREENING METHODS

Eight endpoints will be described in the first phase of toxicity evaluation. The endpoints are chemical/physical properties, existing toxicity literature, preliminary "use" scenarios, qualitative/quantitative structure activity relationships, *in vitro* screening, acute irritation, acute toxicity, and genotoxicity of the halon replacement candidate. Animal or cell studies are not required for the first four endpoints, but they are necessary to determine and evaluate a candidate's potential to cause irritation, toxicity, or mutagenicity, unless this information is already reported in the literature. Figure 1 is a flow diagram of toxicity testing during Phase 1. Details follow on the approach and direction to be taken.

Figure 1. Flow Diagram - Phase 1 Toxicity Screening Methods



General Chemical/Physical Properties

Chemical structure is the necessary basis for chemical identity. The Chemical Abstracts Service (CAS) registry number is used most frequently to associate chemical structure with chemical identity. If a chemical's structure is unknown, analytical methods, such as mass spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and gas chromatography may provide valuable information to establish chemical structure. Experimental data for physical-chemical properties (i.e., physical state, melting point, boiling point, vapor pressure, solubility, octanol-water partition coefficient, viscosity, etc.) are necessary for characterization of the chemical. These data may be obtained experimentally or by use of computational chemistry tools. In general, the physical-chemical properties of a chemical dictate the likely route of human exposure (e.g., inhalation route for highly volatile chemicals and gases) and, thus, the likely choice of route of administration of the chemical for toxicity testing in laboratory animals. Solubility properties are especially important for liquid or solid chemicals that may require a vehicle for dosing either animals or cell/tissue preparations. Some assessment of the stability of the chemical under long-term storage conditions and as a prepared stock solution is necessary to assure meaningful testing results. Nyden and Skaggs (1999) discuss additional physical-chemical properties that are most relevant to halon replacement candidates. These include environmental impact properties (e.g., ozone depletion potential, global warming potential, and atmospheric lifetime) and materials compatibility (e.g., interaction of the halon replacement candidate with metals, gaskets and lubricants, and the compatibility of the candidate and its combustion by-products with potentially exposed weapons systems).

Existing Toxicity Literature

Given the complexity of health endpoints and the expense in conducting toxicology testing, a literature search on the halon replacement candidate or structurally related analogs to the candidate is extremely useful. Valid analogs should have close structural similarity and the same functional groups. Structurally related chemicals are likely to have similar physical-chemical, environmental, and toxicological properties or follow a predictable pattern of effects. Similarities of chemicals may be based on the following list adopted from http://www.epa.gov/chemrtk/categuid.htm

- A common functional group (e.g. aldehyde, epoxide, ester, etc.); or
- The likelihood of common precursors and/or breakdown products, via physical or biological processes, which result in structurally similar chemicals; and
- An incremental and constant change across a group of structurally related chemicals.

Nyden & Skaggs (1999) present a straightforward, yet thorough approach for conducting a literature search to assess toxic properties of candidate fire suppressants. Most database services are free of charge, though a cost may be incurred when ordering reports and documents. The importance of a thorough literature search cannot be underestimated, since the information obtained will provide overall guidance and direction on all subsequent toxicity testing. Information gained from a literature search may eliminate the need for selected toxicity tests, thus reducing the overall cost of testing.

Preliminary "Specific Use" Scenarios

In general, as halon replacement candidates are being developed, they are being investigated as potential total flooding agents or streaming agents. Some candidates may also be considered for the replacement of other chlorofluorocarbons (CFCs) that are currently in use for other purposes (e.g., refrigerants, solvents, etc.). Knowing the potential end-use of the replacement chemical helps define the preliminary exposure scenario. This information, in turn, helps guide the type of toxicity testing that would be of greatest benefit for determining health consequences. For example, a replacement for a total flooding agent suggests an exposure scenario of high concentration (i.e., fire extinguishing levels) for brief periods of time (i.e., few minutes) under static air (i.e., defined volume) conditions. A replacement for a streaming agent suggests a very different exposure scenario, where specific use conditions include dynamic air (i.e., outdoors) movement. In this case, chemical concentrations in the breathing zone of the user may be only moderate in value. Examples of how this information applies to toxicity testing strategy follows in subsequent sections of this document. Keep in mind that there are several more exposure scenarios that have to be considered by regulatory agencies when assessing potential human health risk. For example, the accidental release of a chemical during normal handling, transportation, or storage procedures is often evaluated for potential harm.

Qualitative/Quantitative Structure Activity Relationships

Quantitative structure-activity relationships (QSARs), based on scientific judgements by experienced toxicologists, may be used as an integral part of health hazard characterization. This approach relies on the toxicologist or chemist being able to fit the new chemical into a category of existing chemicals because of similarities in molecular structure or chemical functionality. In order for this approach to be of value, the existing chemicals category or close structural analog must have its own robust toxicology database and the uncertainty of read-across from the close

structural analogs or the category to the new chemical must be recognized and, if possible, defined.

QSAR analysis has been formalized and computerized for some health endpoints (e.g., cancer, mutagenicity, and teratogenicity) and may be useful with appropriate recognition of the limitations of these programs. A review of the various computerized QSAR programs available commercially is beyond the scope of this document, but this subject has been reviewed recently (Dearden *et al.* 1997). The U.S. EPA has grouped chemical substances with similar physical-chemical, structural and toxicological properties into working categories. Additional candidate categories for the EPA's new chemical review process are proposed by Toxic Substances Control Act (TSCA) New Chemicals Program (NCP) staff based on available data and experience of reviewing premanufacturer's notifications (PMNs) on related substances. These groupings enable the user of the NCP Chemical Categories guidance document to benefit from the accumulated data and decisional precedents within EPA's new chemicals review process since 1987, in order to identify areas of health hazard concern. Currently, there are 51 chemical categories listed in the table of contents of the EPA document, the detailed summaries of which may be found at URL: http://www.epa.gov/opptintr/newchms/chemcat.htm.

Close structural analogs may provide data for occupational exposure and/or data for health hazard evaluation from occupational experience or epidemiology studies. For close structural analogs that have been used for several years, relevant information may be gained from historical experience with human exposure from normal handling. For many existing chemicals with many years of widespread industrial use, no adverse health effects have been observed. On the other hand, in some cases of overexposure or where unexpected toxicity was discovered, adverse effects in occupational populations have occurred. When they are available, retrospective (or case-control) epidemiological studies for close structural analogs may provide insight as to the potential for certain health effects by the new chemical. Recently, Nyden and Skaggs (1999) prepared a general listing of chemical classes, functional groups, possible toxic endpoints, and references of possible QSARs.

In Vitro Screens

In many cases, QSAR can not be performed without a high degree of uncertainty, and insufficient human health hazard or animal toxicology data are available on close structural analogs of the halon replacement candidate to conduct a preliminary health hazard characterization. In instances

such as those, *in vitro* screening tests may be advisable to develop the data necessary for a preliminary health hazard characterization. However, caution must be exercised when considering this approach, because the role of *in vitro* toxicity testing in chemical hazard characterization has not been formalized by U.S. regulatory agencies. A wide range of *in vitro* screening tests has been developed for toxicology endpoints that are the subject of *in vivo* testing proposed below (e.g., acute irritation tests, acute toxicity tests, 14-day/90-day repeated dose toxicity tests, and reproductive/developmental toxicity tests). Cytotoxicity assays are promoted as suitable models for screening for human toxicity. A listing of proposed *in vitro* methods that may be used to generate data predictive of more definitive *in vivo* toxicology results and human health hazard potential follow.

- Skin irritation → *In vitro* skin corrosivity/irritation testing - CORROSITEX™ or Skin^2 (Botham *et al.* 1994)
- Eye irritation → *In vitro* eye corrosivity/irritation testing - EYETEX™ or HET-CAM (Balls *et al.* 1998)
- Acute toxicity → Cytotoxicity assays using cultured cells (Seibert *et al.*, 1996)

Nyden and Skaggs (1999) prepared a list of *in vitro* methods for a wide variety of toxicity endpoints that may be useful depending on the classes of new chemical replacements.

Acute Irritation Tests

This test applies to chemical candidates that are liquids or solids at ambient conditions. Irritancy of chemical vapors or gases is assessed in a more general manner during acute toxicity testing. The acute irritation test evaluates a chemical for its potential to cause irritation in laboratory animals following a single dose. Tests for skin or eye irritation are commonly performed in the rabbit, due to the rabbit's proven responsiveness to irritants and the large database of chemical irritant information that exists on this species (almost exclusively the New Zealand White). Details of the experimental design that is generally followed in acute eye irritation and skin irritation tests are given in EPA's Office of Prevention, Pesticides and Toxic Substances Health Effects Test Guidelines, commonly referred to as the OPPTS 870 Series (www.epa.gov/docs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/). The specific Series numbers are 870.2400 and 870.2500 for acute eye and dermal irritation, respectively. The information gained from acute irritation testing is used to determine, in part, the hazard of a chemical, i.e., its intrinsic toxicological properties. In general, a toxicologist will place the candidate into one of four toxicity categories based on the results of the test. The Department of

Transportation and other agencies use this approach (hazard classification) to determine precautionary labeling of chemicals.

Before initiating tests for acute irritation, there are some points to consider. Strongly acidic or alkaline chemicals (e.g., pH ≤ 2 or ≥ 11.5) need not be tested for eye or skin irritation, since it is presumed that these candidates will be irritating to mucosal surfaces. Also, results from well validated and accepted *in vitro* or QSAR tests that predict irritation may preclude the need for testing of irritation in animals. However, equivocal results from *in vitro* or QSAR tests would indicate a need to carry out irritation tests in animals. Either the eye or the skin irritation test is recommended, though chemicals do not necessarily give similar results in both tests. As part of the phase 1 screening method, one test should be sufficient to evaluate a candidate's irritancy potential. The cost to perform a skin or eye irritation test using less than 6 rabbits is approximately $1500 to $2000. If a test agent were found to be corrosive to the skin or eyes, then further screening of the test agent may not be warranted due to the seriousness of this potential health hazard. Expert opinion is needed to decide whether to continue toxicity screening or stop. The expected human exposure scenario during or following use of the test agent and the consideration of estimated dose, route of entry, and personnel protective measures are factors likely to be discussed in making a final decision.

Acute Toxicity Tests

The acute toxicity test is considered the cornerstone of the phase 1 screening method. One reason for this importance is that the EPA's SNAP program requires acute toxicity of a substitute chemical as part of its submission package. The purpose of the test is to determine and evaluate mammalian toxicity following a single dose or exposure of the halon replacement candidate. Rats are the preferred animal model. Valuable hazard information is gained from this test. Similar to the acute irritation test, acute toxicity testing results will place the candidate into a toxicity category for the purpose of hazard classification. Keep in mind that lethality, as a single end-point of toxicity, is a weak index for hazard assessment. Determination of several end-points of toxicity and the evaluation and interpretation of all data collected will provide greater confidence in the acute hazard assessment of a chemical. For example, answers to the following questions are especially helpful for toxicity evaluation. Did injury or death occur rapidly after dosing or were they first observed several days post-treatment? Were clinical signs consistent with central nervous system stimulation or depression? Did animals lose body weight or was there a depression in weight gain following dosing? Were lesions observed in organs or tissues

during gross necropsy? Was there recovery of clinical signs and when was it observed? A toxicologist compiles this information in order to describe the acute hazard of the candidate material in question. Once established, chemicals are frequently compared on the basis of their acute toxicity. For example, a replacement candidate for Halon 1301 might produce lethality in a group of exposed rats at vapor concentrations far below that of Halon 1301. The replacement candidate would be considered more potent in producing acute lethality than Halon 1301, but other factors, such as fire extinguishing effectiveness need to be considered when determining risk management procedures and costs.

There are several points to consider before initiating an acute toxicity test. One is the route of administration. Since inhalation is the most likely route of exposure for halon replacement candidates, it is the preferred route. However, inhalation testing is more expensive than other routes of chemical testing because it requires more labor effort to carry out the test. Also, more test material is needed to conduct the test. Good information is gained from acute oral or dermal toxicity tests, but important questions still remain. How much of the test material was absorbed following the oral or dermal route? Are the target organs of toxicity identified following the oral or dermal route applicable to those that would be identified following inhalation exposure? Since it is difficult to answer these questions by current extrapolation methods, more pertinent information is gained by performing an acute inhalation test.

Inhalation exposure of chemicals can be in the form of gas or particulate matter (liquid droplets or solid particles). Selection of the most appropriate physical state for inhalation testing is simply a matter of considering the "specific use" scenarios that were described previously. One issue that needs to be dealt with for aerosol or dust inhalation studies is particle size. The animal inhalation exposure needs to contain particles that are respirable. For rats, this is on the order of one micron diameter. There are many factors that determine particle size, such as physical properties of the chemical candidate, exposure concentration, and particle generators. In some cases, the generation of respirable particles is very difficult to attain. Also, there may be discussion that the halon candidate would not be used or perhaps effective with such small particle sizes. However, the value of inhalation toxicity testing is diminished considerably if the distribution of particle sizes generated for toxicity testing of a chemical candidate does not fall within the range considered respirable for the animal species tested.

The "Limit Test". Exposure duration and chemical concentration are the two most important points to consider in the experimental design of an acute inhalation toxicity test. The current OPPTS Series 870.1300 guidelines recommend 4 hours duration after equilibration of the chamber concentration. Selecting a target exposure concentration that produces a defined response in animals may be difficult, but in the absence of any toxicity data on the halon replacement candidate, EPA allows one to consider using the "limit test" approach. One purpose of the limit test is to minimize the number of laboratory animals needed for testing, yet provide an estimate of a chemical's acute hazard. Under DoD Directive #3216.1, stringent polices dictate that the most conservative approaches must be taken with regard to the use of laboratory animals in research programs. The need to spend time and resources to obtain a once traditional "LC_{50} value" for a halon replacement candidate is discouraged. In the limit test, five male and five female animals (usually rats) are exposed to a chemical concentration of 2 mg/L for 4 hours, or where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration. If no lethality is observed during exposure or for 14 days post-exposure, no further testing for acute inhalation toxicity is needed. Chemicals that do not cause lethality in a limit test evaluation fall into EPA's least hazard toxicity category. If lethality is observed, additional 4-hour exposures at lower concentrations are strongly recommended, unless the test substance is no longer a candidate for consideration due to its acute hazard. Depending on the incidence of lethality observed at 2 mg/L, a concentration of 1 mg/L or 0.2 mg/L is suggested for additional animal exposures. The goal of these exposures would be to determine a non-lethal or low (<20% incidence) lethal 4-hour concentration value of the test substance. This information is useful for comparing the acute inhalation hazard of chemicals and for designing repeated exposure experiments (Phase 2 – Toxicity Testing Methods). The cost to perform an acute inhalation toxicity "limit test" is approximately $7,500 to $10,000. Costs can be quite variable depending on the analytical method chosen for study and/or the time to develop new analytical methods, if necessary.

The "Specific Use" Scenario Test. In general, the "specific use" scenario for a halon replacement candidate is for a few minutes only of exposure duration, but at a concentration much higher than the limit test value of 2 mg/L. One might ask what would be the hazard assessment for a candidate under these conditions? Exposures that simulate real-world conditions do help characterize the overall risks to human health, and risk characterization is a subject of concern for regulatory purposes. Thus, this type of inhalation exposure is recommended for halon replacement candidates. The goal of this test is to determine the potential for adverse

effects following a single, high concentration exceedingly short exposure. A logical first choice for test atmosphere concentration and exposure duration would be the "worse-case" exposure scenario defined previously under the topic of preliminary "specific use" scenarios. The concentration of the test substance atmosphere should not be so high that oxygen content of at least 19% could not be maintained throughout the exposure duration. Exposure duration of 30 minutes or less is difficult to control via whole-body test systems, thus nose-only exposure designs are recommended. Once the concentration and duration are selected, the study design is identical to that of the "limit test" described previously. Additional animal exposures would need to be performed, if toxicity were observed, and more acute inhalation hazard data were desired. At this point in the testing scheme, a decision would have to be made to carry out exposures at lower concentrations (same duration) or follow the OPPTS guidelines of a 4-hour exposure duration, starting with the limit test concentration of 2 mg/L. Making an assumption that one can simply apply a concentration x time relationship (Haber's Law) to acute inhalation toxicity data can be erroneous (discussed below).

As mentioned previously, acute toxicity data may be obtained for some test substances, such as liquids and solids stable at ambient conditions, by the oral route. The same general concepts described for the inhalation route apply, i.e., selection of limit test and LD_{50} determination, and are discussed in the EPA Health Effects Test Guidelines OPPTS Series 870.1100. The limit test value is 2,000 mg/Kg (body weight). Careful consideration should be given to the choice of vehicle, if necessary, and the maximum volume of liquid to be administered at one time. Recommendations are provided in the EPA Guidelines.

Similar to the results of skin/eye irritation determination, test substances showing highly toxic characteristics (e.g., death, violent convulsions) at small or moderate doses may not warrant further consideration for toxicity screening. Again, expert opinion is needed to decide whether to continue toxicity screening or stop. The "specific use" scenario and the consideration of estimated dose, route of entry, and personnel protective measures are factors likely to be discussed in making a final decision.

Genotoxicity Tests (Phase 1)

Genotoxicity assays detect and estimate the genetic hazard of chemicals. The purpose of the assays is to screen for possible mammalian mutagens and carcinogens. There are a wide variety of tests to consider when selecting an assay to determine a candidate's potential to cause genetic

damage. For this reason, a battery of three or more assays is commonly performed as a first tier approach for assessing genotoxicity. However, to gain as much preliminary information as possible with minimal cost, only one assay is recommended as part of Phase 1 – Toxicity Screening Methods. The *Salmonella typhimurium* reverse mutation assay, commonly referred as the Ames assay, is probably the most widely used genotoxicity test for assessing mutagenicity. Results provide information on whether the test substance causes point mutations in the genome of this bacterial organism. Details of the assay are available in OPPTS Series 870.5265. Currently, four test strains (TA 1535, TA 1537, TA 98, and TA 100) are designated for testing, but a fifth strain (TA 102) is recommended (OPPTS Series 870.5100). Each strain is tested in the presence and absence of a metabolic activation system with appropriate positive control reference substances to ensure the efficacy of the activation system. A preliminary "range-finding" experiment is required to determine the upper limits of a candidate's concentration that will produce cytotoxicity and/or, for relatively insoluble chemicals, the limits of solubility. A maximum of 5 mg test substance per plate is acceptable by EPA.

The Ames assay is fairly straightforward for liquid or solid test materials at ambient conditions. The assay is more complicated for gas or vapor halon replacement candidates. This is because of the time and skill required exposing bacteria cultures to the test substance. Exposure chambers may have to be designed or modified. Vapor generation systems and analytical methods to measure the exposure concentration of the test substance have to be developed and implemented. A determination of test substance solubility is desirable, particularly when no cytotoxicity is observed in the "range-finder" experiment. The cost to perform the Ames assay for liquid or solid test materials is about $7,000. For gas or vapor exposures, the cost can be an additional $10,000 depending on the development and implementation of analytical methods. However, to keep cost at a minimum during Phase 1 testing of volatile halon replacement candidates, a protocol for the genotoxicity screen of volatile compounds was prepared (Appendix A). In this protocol, two sensitive tester strains of *Salmonella* (TA100 and TA102) are used to examine potential genotoxicity. Tedlar® bags are employed for preparation of vapor test atmospheres and exposure of bacteria.

DECISION POINT 1

The primary objective for establishing decision points in the strategy for safety evaluation of potential halon replacement chemicals is to decide if more resources and funds should be expended to obtain more data to improve confidence and reduce uncertainty in assessing risk to

human health. Phase 2 – Toxicity Testing is more time consuming than Phase 1 – Toxicity Screening Methods and requires considerable more funds to carry out testing. The information gained from Phase 1 – Toxicity Screening Methods includes chemical/physical properties, existing toxicity literature, preliminary "use" scenarios, qualitative/quantitative structure activity relationships, *in vitro* screens, acute irritation, acute toxicity, and genotoxicity of the halon replacement candidate. The value of this information in terms of human health hazard is noteworthy, but relatively small in amount (unless the toxicity literature database is extensive and of high quality). In general, hazard classification, precautionary labeling, and handling measures (i.e., use of personal protective equipment) can be recommended for a test substance using data from Phase 1 – Toxicity Screening Methods. Further, the intrinsic acute hazard of the halon replacement candidate can now be compared to chemicals with known properties of irritation, acute toxicity, and mutagenicity. For example, the acute toxicity and mutagenicity hazard of Halon 1211 (bromochlorodifluoromethane), Halon 1301 (bromotrifluoromethane), CFC 11 (trichlorofluoromethane), and CFC 12 (dichlorodifluoromethane) are frequently compared to that of a replacement candidate (Table 1).

Table 1. Acute Toxicity of Halon 1211, Halon 1301, CFC 11, and CFC 12

Halon/CFC	Finding
Halon 1211	Rat LC_{50} (4-hr) = 31,000 ppm); Rat LC_{50} (15-min) = 200,000 ppm
Halon 1211	Ames test – negative in 4 strains; positive in 1 strain
Halon 1301	Rat LC_{lo} (4-hr) > 800,000 ppm
Halon 1301	Ames test – negative in 5 strains
CFC 11	Rat LC_{50} (4-hr) = 26,200 ppm); Rat LC_{50} (15-min) = 130-150,000 ppm
CFC 11	Ames test – negative
CFC 12	Rat LC_{50} (4-hr) > 800,000 ppm
CFC 12	Ames test – negative

Another useful table is the toxicity categories assigned to products by EPA to assist in the selection of personal protective equipment required under the Worker Protection Standard set forth in 40 CFR 170.240 (Table 2). Halons 1211 and 1301 and CFCs 11 and 12 would fall into Category IV. This provides also a comparison perspective for replacement candidates that were tested by oral or dermal routes of administration.

Table 2. Toxicity Categories*

Type of Study	Category I (most hazard)	Category II	Category III	Category IV (least hazard)
Acute Oral LD_{50}	Up to and including 50 mg/Kg	>50 through 500 mg/Kg	>500 through 5000 mg/Kg	>5000 mg/Kg
Acute Dermal LD_{50}	Up to and including 200 mg/Kg	>200 through 2000 mg/Kg	>2000 through 5000 mg/Kg	>5000 mg/Kg
Acute Inhalation LC_{50}	Up to and including 0.05 mg/L	>0.05 through 0.5 mg/L	>0.5 through 2 mg/L	>2 mg/L
Eye Irritation	Corrosive or corneal involvement or Irritation >21 days	Corneal involvement or irritation clearing in 8-21 days	Corneal involvement or irritation clearing in ≤7 days	Minimal effects clearing in ≤24 hours
Skin Irritation	Corrosive	Severe irritation at 72 hours	Moderate irritation at 72 hours	Mild or slight irritation

*EPA Health Effects Test Guidelines OPPTS 870.1000 Acute Toxicity Testing – Background

The Department of Transportation has toxicity categories for assigning hazardous materials to hazard zones and packing groups (CFR, Title 49-Transportation, Part 173). Information needed for group assignment of gases and dusts/mists includes the material's 1-hour LC_{50} value in young adult albino rats (Tables 3 and 4, respectively). For volatile liquids, the saturated vapor concentration at ambient conditions is needed (Table 5). Packing groups for chemical mixtures are based on mole fractions of the individual components (CFR 49, Part 173.133(b)).

Table 3. Hazard Zone Assignment for Poisonous Gases*

Hazard Zone	Inhalation Toxicity
A	$LC_{50} \leq 200$ ppm
B	$LC_{50} > 200$ ppm and ≤ 1000 ppm
C	$LC_{50} > 1000$ ppm and ≤ 3000 ppm
D	$LC_{50} > 3000$ ppm and ≤ 5000 ppm

*CFR 49, Part 173.116

Table 4. Packing Group Assignment for Poisonous Dusts and Mists*

Packing Group	Inhalation Toxicity
I	$LC_{50} \leq 0.5$ mg/L
II	$LC_{50} > 0.5$ mg/L and ≤ 2 mg/L
III	$LC_{50} > 2$ mg/L and ≤ 10 mg/L

*CFR 49, Part 173.133

Table 5. Packing Group Assignment for Poisonous Liquids Based on Inhalation of Vapors*

Packing Group	Vapor Concentration and Toxicity
I (Hazard Zone A)	V** $\geq 500\ LC_{50}$ and $LC_{50} \leq 200$ ml/m^3***
I (Hazard Zone B)	$V \geq 10\ LC_{50}$; $LC_{50} \leq 1000$ ml/m^3; and the criteria for Packing Group I, Hazard Zone A are not met
II	$V \geq LC_{50}$; $LC_{50} \leq 3000$ ml/m^3; and the criteria for Packing Group I, are not met
III	$V \geq 0.2\ LC_{50}$; $LC_{50} \leq 5000$ ml/m^3; and the criteria for Packing Groups I and II, are not met

*CFR 49, Part 173.133

**V is the saturated vapor concentration in air of the material in ml/m^3 at 20°C and standard atmospheric pressure

***ml/m^3 = ppm = mg/L x 24,450/MW, where MW = molecular weight of the material and 24,450 is the molar volume of air in milliliters at 25°C and 760 mm Hg

Concentration x Time (C x T) Relationships. In 1924, Haber described a relationship between concentration, time, and animal death that became known widely as "Haber's Law" (Haber, 1924). Haber's Law states that the product of the concentration (C) and time (T) of exposure required to produce a specific physiological effect is equal to a constant (k), $C \times T = k$. Recently, attention has been focused on a similar postulate, $C^n \times T = k$, where the exponent n is derived from probit regression coefficients similar to that described by ten Berge *et al.*, 1986. The $C^n \times T = k$ relationship may be a better predictor for mortality of irritants and systemically acting vapors and gases. Clearly, there are limitations to both relationships, but for many volatile chemicals, these relationships are applicable especially when lethality is considered the effect and time of exposure is acute and within a narrow range, such as several minutes to a few hours. Halons, however, may be a class of chemicals that does not follow a $C \times T = k$ relationship for the endpoint of lethality in laboratory animals. Using the data in Table 1 for Halon 1211 and applying Haber's Law, the product of C x T ranges from 50,000 to 124,000 ppm•hour for the 15-minute and 4-hour LC_{50} values, respectively. This difference is approximately 2.5-fold. Halon 1202 has 15-minute, 2-hour, and 7-hour LC_{50} (or approximate LC_{50}) values of 110,000, 40,000, and 20,000 ppm, respectively, in laboratory rats (DuPont, 1978). The ppm•hour product ranges from 27,500 to 140,000, an approximate 5-fold difference. Halon 2402 has 15-minute and 4-hour LC_{50} values of 120,000 and 55,000 ppm, respectively, in laboratory rats (DuPont, 1985). The ppm•hour product ranges from 30,000 to 220,000, an approximate 7-fold difference. Thus, caution must be exercised in applying C x T relationships for chemicals or chemical classes that don't have databases to support the use of Haber's Law or the ten Berge *et al.* postulate.

Stop or Continue Testing. Expert opinion and advice are recommended to assist in making the decision to either drop a candidate from further testing or to proceed to Phase 2 – Toxicity Testing. Current scientific literature may have data on toxicity tests or studies that aren't the same as those described in Phase 1 – Toxicity Screening Methods. An expert will have to address if acute hazard can be assessed from the existing literature data and compared to other halons. For example, the test substance of concern might have a higher 4-hr LC_{50} value than that of Halon 1211, but is mutagenic in the Ames test in the presence of a metabolic activation system. Many compounds that are positive in the Ames test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Also, how much emphasis should be placed on QSAR results? Hazard interpretation of these types of information requires experience and knowledge. A test substance candidate that is classified as highly acutely toxic (Category I), shows mutagenicity in the Ames test, and lacks additional literature hazard data would likely be

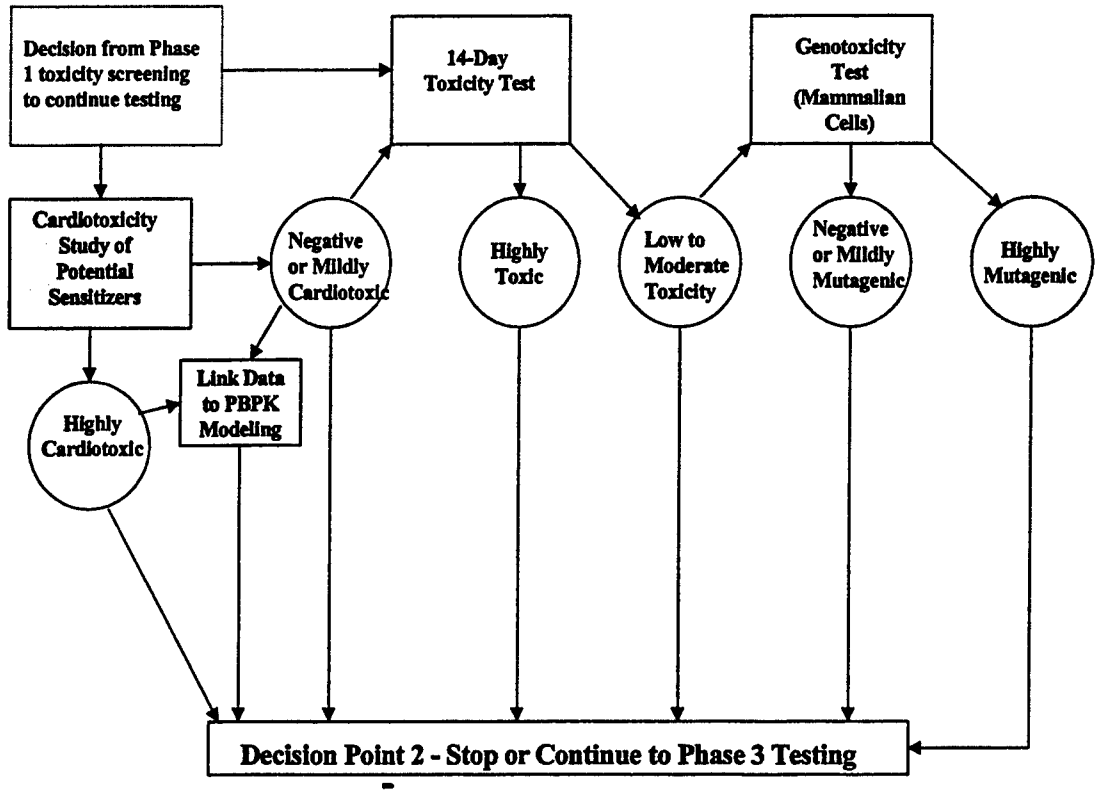
dropped from further testing consideration. However, important in the decision making process is the consideration of preliminary "specific use" scenarios. Since risk is an index of both hazard and exposure, a test substance of high or moderate acute toxicity might be appropriate for unoccupied spaces or nonresidential uses. These "narrowed use" limitations are part of EPA's assessment process for overall risk in the SNAP Program.

Halon replacement candidates that show low order of irritancy and acute toxicity, are not mutagenic in the Ames assay, and do not indicate signs of delayed or cumulative toxicity based on results of QSAR or literature searches, are candidates that will likely proceed to Phase 2 – Toxicity Testing.

PHASE 2 – TOXICITY TESTING

Three endpoints will be described in the second phase of toxicity evaluation. They are cardiotoxicity, repeated dose toxicity, and genotoxicity. Figure 2 is a flow diagram of toxicity testing during Phase 2. Details follow on the approach and direction to be taken.

Figure 2. Flow Diagram - Phase 2 Toxicity Testing



Cardiotoxicity Study

One of the most important factors in assessing the safety of a halon replacement candidate is cardiac sensitization. Cardiac sensitization refers to heart rate arrhythmia experienced after injection of epinephrine and inhalation of the test chemical. The phenomenon of sensitizing the heart to epinephrine is associated with a number of unsubstituted and halogenated hydrocarbons. There are no regulatory testing guidelines for conducting a cardiac sensitization study. However, the experimental design most often followed is that of Reinhardt *et al.*, 1971. Dogs are used as test subjects. One of the most important features of the experimental design is the establishment of cardiac response of each individual dog to epinephrine challenge alone. The typical response consists of a transient increase in heart rate followed by a slowing of heart rate and an increase in the height of the T-wave. In some dogs, multiple ventricular tachycardia occurs. To establish a predictable and minimal (i.e., baseline) cardiac response, epinephrine is administered intravenously at doses ranging from 1 to 12 μg/kg (body weight) at a rate of 0.1 ml/kg. It is not uncommon to find that the baseline response to epinephrine challenge will range from 1 to 12 μg/kg in a small (<10) group of naïve dogs, and that a few dogs from the group will be eliminated from the study due to unpredictable or greater than minimal response to epinephrine. This feature of the experimental design, the concern of the use of canine as an animal model that results frequently in mortality (due to ventricular fibrillation), and the issue of toxicological significance attributed to single animal reponses, have led to considerable debate among scientists and regulators, including the search for alternative methods in assessing cardiotoxicity of replacements to chlorofluorocarbons (Snyder *et al.*, 1997).

The cardiac sensitization study results in determining two critical endpoints for a test chemical, the lowest-observable-adverse-effect-level (LOAEL) and the no-observable-adverse-effect-level (NOAEL). However, these determinations are not calculated or extrapolated from the study data, but are determined simply by the selection of target exposure concentrations defined by the experimental design. The determination of the LOAEL and NOAEL become the key factor in assessing the safety of a halon replacement agent. However, physiologically based pharmacokinetic (PBPK) models have been developed to better define the relationship between acute exposure and cardiosensitization effect (see Linking PBPK Modeling).

From a regulatory perspective (e.g., EPA), obtaining information on the cardiac sensitization potential of a halon replacement candidate is essential, assuming that the replacement candidate has chemical structure and properties analogous to halogenated hydrocarbons. Under EPA's

SNAP, cardiac sensitization LOAELs and NOAELs help define a replacement chemical's use restrictions. For example, when using halon substitutes as total flooding agents the following conditions, which were adopted from an OSHA safety and health standard (29 CFR 1910 Subpart L), generally apply:

- Where egress from a normally occupied area cannot be accomplished within one minute, the employer shall not use the agent in concentrations exceeding its NOAEL.
- Where egress from a normally occupied area takes longer than 30 seconds but less than one minute, the employer shall not use the agent in a concentration greater than its LOAEL.
- Agent concentrations greater than the LOAEL are only permitted in areas not normally occupied by employees provided that any employee in the area can escape within 30 seconds. The employer shall assure that no unprotected employees enter the area during agent discharge.

Halon substitutes being used as streaming agents are restricted to nonresidential uses. EPA's SNAP conditions on agent use are different for substitutes of technologies unrelated to halogenated hydrocarbons or the potential to produce cardiac sensitization, such as inert gases, water mist, or powdered aerosols.

The cost of testing one compound for cardiac sensitization is between $40-65K, and the cost of running two to three test materials is about $70-80K due to the fact that set-up costs would be done only once and study animals may be used repeatedly among the testing of different agents. The time to complete a cardiotoxic study is approximately two months. Only a few laboratories perform this testing—those with both inhalation and canine capabilities. One of the reasons the number of laboratories is limited is that physiological equipment and personnel to perform and interpret electrocardiograms are necessary.

As mentioned previously with test substances that were either corrosive to the skin and eyes or highly toxic following single small doses, test materials that are potent cardiac sensitizers may not warrant further consideration for toxicity evaluation. Expert opinion is needed to decide whether to continue toxicity testing or stop. The "specific use" scenario and the consideration of estimated dose, route of entry, and personnel protective measures are factors likely to be discussed in making a final decision. An additional consideration, if applied, is the results and

interpretation from physiologically based pharmacokinetic (PBPK) modeling of the test substance and its cardiac sensitization response.

Linking PBPK Modeling to Cardiac Sensitization Potential. Note: excerpts are taken from Vinegar and Jepson (1996). As mentioned previously, human exposure to halon replacement chemicals may be regulated on the basis of cardiac sensitization potential. After a range of chemical concentrations has been evaluated using the cardiac sensitization protocol, the dose-response data can be used to establish the NOAEL and LOAEL. Utilization of the animal testing data for assessing potential risks to humans requires development of appropriate risk assessment methods. These methods must address the unusual exposure circumstances involved in the use of chemicals as fire suppressants. In particular, potential exposures would be for relatively brief, but varying, periods of time at concentrations high enough to effectively extinguish fires. Egress times need to be established for people occupying a facility at the time of chemical agent discharge. Currently, the LOAEL determined in dogs has been applied directly by the EPA in evaluating acceptable use and allowable exposure limits for humans. Establishing egress times from the 5 min exposure LOAEL requires careful consideration of the relationship between exposure concentration, duration, and the temporal aspects of the biological response. Under steady state conditions, the concentration in blood and tissues would be constant regardless of the exposure duration. With pre-steady state conditions, concentrations vary with time. Using the quantitative relationship between inhalation exposure at the LOAEL and the resulting concentration of chemical in blood is a way to make scientifically based decisions about the egress times. Cardiac sensitization is considered to be a function of the concentration of parent chemical in heart tissue which under perfusion limited conditions is proportional to parent chemical in blood. Therefore, the approach described herein involves estimating a target level defined as the chemical concentration in human blood that is achieved after 5 min of inhalation exposure at the chemical LOAEL as determined in dogs.

A PBPK model provides the quantitative link between exposure concentration and blood levels of chemical achieved following inhalation. The PBPK model is a mathematical description of the uptake, distribution, metabolism, and elimination of a chemical in the species of interest. The physiological compartments that compose the model are based on appropriate physiological and anatomical properties for the species of interest as well as the chemical specific properties of the test compound. The use of PBPK models for kinetic description of chemical interaction with

biological systems has been well represented in the scientific literature and is widely accepted by the scientific community as a tool for risk assessment.

14-Day Dose Range-Finding Study

This is the first study in the proposed series of toxicity testing to assess and evaluate the potential toxic characteristics of a replacement chemical following *repeated* doses (or exposures). Though important data can be obtained from a 14-day study, it is often performed as a "dose range-finder" for the 90-day study (see Phase 3 – Toxicity Testing). Thus, the 14-day study is streamlined to use fewer animals, take less time to complete, and cost about 20% of that of a 90-day study. Since the 14-day study is considered primarily to be a dose range-finder for longer term studies, the experimental design can be flexible. The following list provides some guidelines in experimental design that maximize the obtainment of information and minimize costs.

- Use adult rats as the species for testing; 5 males and 5 females per dose group
- Use 3 or 4 dose groups; 2 or 3 treated and one untreated (control)
- For inhalation studies, expose animals 6 hr/day, 5 days/wk for 2 weeks; for other routes of administration, dose animals for 13 continuous days
- Endpoints should include daily clinical observations, body weight measurements every 2-3 days, routine hematology and blood biochemistry determinations (at conclusion of the study), gross necropsy, and select organ weight measurements (e.g., liver, lungs, kidneys, adrenals, testes, brain)
- Preserve select organs and tissues in neutral buffered formalin for possible histopathological examination

Following these guidelines will result in providing useful toxicological information at a cost of less than $50,000 and a period of time less than 45 days. The selection of doses (or exposure concentrations for inhalation studies) is important and requires careful consideration. The purpose of the highest dose is to produce some toxicity, even mortality. The intermediate and lowest doses may also produce toxicity, though usually less severe. It is not necessary to determine a NOAEL, because this determination is reserved for the longer term, more detailed (with endpoints) 90-day study. Two suggestions follow on selecting a high dose for a 14-day study. For chemicals that appear to be mildly toxic, i.e., categories III and IV (Table 2), select EPA's "limit test" dose for 90-day studies, unless one suspects that human exposure might be

higher. The limit test value is 1 mg/L via the inhalation route, and 1,000 mg/kg body weight for oral administration. Consideration should be given also to minimize cost and select only two treated groups for the 14-day study. For chemicals that appear to be more potent, i.e., categories I and II (Table 2), start with an exposure concentration (or dose) that is 50-75% of the acute LC_{50} or LD_{50} value. Three treated groups are recommended with a concentration range of approximately one order of magnitude between the highest and lowest dose groups.

Genotoxicity Tests (Phase 2)

A bacterial cell system was recommended for genotoxicity screening in Phase 1 – Toxicity Screening Methods. However, additional tests need to be carried out to provide greater confidence in predicting potential genotoxicity health hazard. In this phase of testing, assessment of genotoxicity involves a mammalian cell system. There are several assays to consider, but the two most common are the *in vitro* gene mutation assays in Chinese hamster ovary (CHO) cells and mouse lymphoma (L5178Y) cells. EPA guidelines for these tests are available (OPPTS 870.5300). Each test costs $17-22K for liquid or solid test materials and takes 2 to 3 months to complete. Again, if the halon replacement chemical can be tested as a vapor only, the cost and time to complete each assay will increase. Two references for testing volatile substances in CHO cells are Krahn *et al.* (1982) and Zamora *et al.* (1983). A reference for testing vapor of halon replacement candidates in L5178Y cells is Dodd *et al.* (1997). Only one assay needs to be selected for chemical testing, because both assays give similar type of genotoxicity information. The choice of an assay depends primarily on the historical background and familiarity of the laboratory performing the test. Similar to the Ames assay described previously, a preliminary "range-finding" experiment is performed to determine the upper limits of a candidate's concentration that will produce cytotoxicity. The use of an exogenous source of metabolic activation to mimic mammalian *in vivo* conditions is part of the study design.

DECISION POINT 2

Information gained from Phase 2 – Toxicity Testing includes a test candidate's potential for cardiac sensitization, short-term repeated exposure toxicity, and genotoxicity in a mammalian cell system. By now, a much more realistic profile of the toxicity of the halon replacement should be available. The value of this information in terms of human health hazard is noteworthy, especially with regard to cardiac sensitization. As discussed previously, the intrinsic hazard of the halon replacement candidate can be compared with other chemicals, such as Halon 1211, Halon 1301, CFC 11, and CFC 12 (Tables 6, 7, and 8).

Table 6. Cardiac Sensitization NOAELs and LOAELs (Canine)

Halon/CFC	NOAEL (5-min)	LOAEL (5-min)	Comments
Halon 1211	5,000 ppm	10,000 ppm	EC_{50} = 19,000 ppm
Halon 1301	50,000 ppm	75,000 ppm	EC_{50} = 200,000 ppm
CFC-11	3,200 ppm	3,500 ppm	EC_{50} = 9,000 ppm
CFC-12	40,000 ppm	50,000 ppm	EC_{50} = 77,000 ppm

N.A. = not available

EC_{50} = A calculated concentration that represents an effect in 50% of the animals in the study group

Table 7. Short-Term Repeated Exposure Toxicity Data (Rat)

Halon/CFC	Exposure Duration	NOAEL or LOAEL
Halon 1211	6 hr/day x 15 days	10,000 ppm LOAEL 3,300 ppm NOAEL
Halon 1301	2 hr/day x 15 days	500,000 ppm NOAEL
CFC-11	1-hr (twice)/day x 15 days	50,000 ppm LOAEL 25,000 ppm NOAEL
CFC-12	3.5 hr/day x 20 days	100,000 ppm NOAEL

Table 8. In Vitro Mammalian Cells Genotoxicity Data

Halon/CFC	InVitro Mammalian cells
Halon 1211	Negative
Halon 1301	N.A.
CFC-11	Negative
CFC-12	Negative

N.A. = not available

Stop or Continue Testing. Expert opinion and advice are recommended to assist in making the decision to either drop a candidate from further testing or to proceed to Phase 3 – Toxicity Testing. Since the results of cardiac sensitization testing is very important to the EPA with respect to the candidate's use in occupied areas, this information will be the most critical to evaluate. Results of PBPK modeling will help clarify the association between safe concentration and egress time. EPA will most likely designate a potent cardiac sensitizer for use in unoccupied areas only. Thus, potent cardiac sensitizers may continue to have potential use; however, the concern for issues of safe transportation, storage, and handling of a halon replacement candidate that is restricted for use in unoccupied areas remains to be addressed. In this phase of evaluation, results of repeated exposure toxicity studies begin to play a more important role in predicting health hazard. In the 14-day dose range-finding study, indicators of toxicity include unusual or persistent clinical signs (e.g., decreased activity), decreases in body weight, abnormalities in blood hematology or serum chemistries, alterations in organ weights, and the presence of gross organ/tissue lesions. Depending on the nature and severity of the effect and the exposure concentration required to elicit the effect, a decision to stop or continue testing can be made. Similar to the previous discussion on interpretation of genotoxicity results from the Ames test, a positive finding of mutagenicity in an *in vitro* mammalian cell system does not indicate absolute certainty that a chemical will cause cancer in humans. For greater confidence in predicting genotoxicity consequences in humans, more specific testing is required.

Halon replacement candidates that are not potent cardiac sensitizers, show low order of toxicity in the repeated dose range-finding study, and are not mutagenic in mammalian cell systems, are candidates that will likely proceed to Phase 3 – Toxicity Testing.

PHASE 3 – TOXICITY TESTING

Only those candidates that are seriously being considered for halon replacement should proceed with Phase 3 - Toxicity Testing, due to the high cost in performing these studies. Two tests will be presented; the 90-day subchronic study and an *in vivo* genotoxicity study that will complete the "battery of tests" for genotoxicity potential.

90-Day Subchronic Study

EPA considers a single, well-conducted, subchronic mammalian bioassay by the appropriate route as a minimum database for estimating a reference dose (RfD) or reference concentration (RfC). Rats are the most common mammalian species chosen for investigation. The RfD and RfC are defined by EPA as an estimate (with uncertainty ranging approximately an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. An important component of the RfD is the NOAEL of the toxicology study that evaluates the most important biological endpoints of the chemical in question. In many cases, the results of the 90-day study provide the NOAEL used for setting RfDs and RfCs, because target organs and systemic effects are identified and evaluated in the study.

The biological endpoints of a 90-day study include daily clinical observations; measurements of body weight, food consumption, and organ weights; hematological, serum chemistry, and urine determinations; gross necropsy; and histopathology of numerous tissues and organs. Depending on the toxicity profile to-date of the halon replacement to be evaluated, assessments of neurotoxicity, reproductive toxicity, and immunotoxicity can become part of the 90-day study design. EPA guidelines are available for the basic 90-day study designs (OPPTS 870.3465 for inhalation route of administration and OPPTS 870.3100 for the oral route in rodents). The cost of basic 90-day study designs ranges from $130,000 (oral) to approximately $200,000 (inhalation), but add-ons for specific assessments can increase the cost another $40,000 to $80,000.

Selection of doses or exposure concentrations for the 90-day should be fairly straightforward, because the results of the 14-day dose range-finder study (phase 2) are the primary determinants. It is important that the highest dose selected for the 90-day study cause some adverse effects, and that the lowest dose selected produce no adverse effects, i.e., a NOAEL. A dose range of an order of magnitude (e.g., 10,000 ppm to 1,000 ppm) is not unusual for 90-day studies.

Genotoxicity Tests (Phase 3)

As discussed previously, the prediction of a chemical to produce cancer in humans from the results of short-term testing in cell culture systems is more reliable when information is available from several genotoxicity test systems, i.e., a "battery of tests." The conducting of an *in vivo* mammalian cell genotoxicity test will complete the "battery of tests" that is often used to

determine genotoxicity potential. An *in vivo* mammalian cell test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes although these may vary among species, among tissues, and among genetic endpoints. The *in vivo* mammalian cell system used frequently in a genotoxicity test battery is the mouse micronucleus assay. The mouse micronucleus test detects the damage of chromosome or mitotic apparatus caused by a chemical in immature (polychromatic erythrocyte; PCE) cells of treated animals. Micronuclei are believed to be formed from chromosomes or chromosome fragments left behind during cell division. After cell division, these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. So the micronucleus test can serve as a rapid screen for clastogenic agents and test materials that interfere with normal mitotic cell division, effecting spindle fiber function or formation. The assay is based on the increase in the frequency of micronucleated PCEs in bone marrow of the treated animals.

General guidelines to be followed in the mouse micronucleus assay are described in EPA's Health Effects Test Guidelines OPPTS 870.5395. More specifically, the assay is conducted using animals of both sexes and 3 to 4 concentrations of the test agent or negative control (e.g., air-only exposed or saline dosed). Treatment of animals is either by inhalation (volatile test chemicals) or by gavage for three consecutive days. A positive control agent, cyclophosphamide, dissolved in physiological saline, is given by intraperitoneal injection as a single dose. Twenty-four hours following the last exposure/dose, mice are sacrificed, and bone marrow cells are collected and processed. The frequency of micronucleated cells is observed by counting 2000 PCEs per animal. Animal mortality and the ratio of PCE to mature erythrocytes (normochromatic erythrocyte; NCE) are used as indicators of test agent toxicity. The difference in the micronucleated PCE frequency and the ratio of PCE /NCE between treated and control animals are statistically evaluated by Chi-square analysis, and the dose dependent response is examined by linear regression. A test agent is considered to have elicited a positive response in the mouse micronucleus test if there is a dose-related increase in micronuclei and if one or more of the doses induces a statistically significant ($p < 0.05$) increase in micronuclei induction. The assay typically costs $20,000 to $35,000, depending on the route of administration of the halon replacement to be tested.

DECISION POINT 3

Information gained from Phase 3 – Toxicity Testing includes a chemical candidate's potential for intermediate-term (90-day) repeated exposure toxicity and genotoxicity in an *in vivo* mammalian cell system. Much more confidence of the toxicity profile of the halon replacement is now available for predicting human health hazard. As discussed previously, the intrinsic hazard of the halon replacement candidate can be compared with other chemicals, such as Halon 1211, Halon 1301, CFC 11, and CFC 12 (Tables 9 and 10).

Table 9. 90-Day Repeated Exposure Toxicity Data (Rat)

Halon/CFC	NOAEL or LOAEL	Target Organ of Concern for Toxicity
Halon 1211	N.A.	-
Halon 1301	23,000 ppm NOAEL*	Respiratory tract*
CFC-11	1,000 ppm (24 hr/day) NOAEL	Cardiotoxicity; CNS (depression)
CFC-12	10,000 ppm NOAEL	CNS (depression)

N.A. = not available

CNS = central nervous system

*18-week study

Table 10. In Vivo Mouse Micronucleus Assay Data

Halon/CFC	InVivo Mouse Micronucleus Result
Halon 1211	50,000 ppm NOAEL (6 hr/day x 1 day)
Halon 1301	N.A.
CFC-11	N.A.
CFC-12	N.A.

N.A. = not available

Stop or Continue Testing. Expert opinion and advice are recommended to assist in making the decision to either drop a candidate from further testing or to proceed to Phase 4 – Toxicity Testing. By now, a considerable amount of toxicity information is available on a halon replacement candidate and estimates of RfCs or RfDs can be made. Again, the RfC and RfD are defined by EPA as an estimate (with uncertainty ranging approximately an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. Histopathology data from the 90-day study and results from the genotoxicity test battery provide moderate to strong confidence in predicting carcinogenic potential of a chemical. However, important biological endpoints such as developmental toxicity and reproductive toxicity remain to be addressed more thoroughly.

The decision to begin Phase 4 – Toxicity Testing implies that a chemical candidate is being seriously considered as a halon replacement. Occupational exposure is likely due to industrial manufacturing, processing, distribution, and storage of the new chemical. The general public may be at risk as well. Thus, it becomes imperative to determine and evaluate the potential of a new chemical that is being considered for industrial production to cause more specific endpoints of toxicological concern. Phase 4 – Toxicity Testing addresses some of these concerns.

PHASE 4 – TOXICITY TESTING

As discussed previously, only those candidates that are seriously being considered for manufacture and use should proceed with Phase 4 – Toxicity Testing, due to the high cost in performing these studies, especially by the inhalation route of exposure. Two tests will be presented in detail (developmental and reproductive toxicity studies), but other tests will be listed for completeness.

Developmental Toxicity Study

Developmental toxicity is any adverse effect observed in the fetus/neonate induced during the period from conception through puberty. The major types of developmental toxicity are embryo-lethality, structural abnormalities, altered growth and functional deficiencies. Chemical effects on the developing fetus may be mediated through toxicity in the parents. These effects are not generally considered to be developmental toxicity. However, effects in the fetus that result from the direct interaction of the chemical with developmental processes are attributed to developmental toxicity and generally raise the level of concern about chemical hazard.

A developmental toxicity study in rodents typically costs $100,000, requires approximately eight months from initiation to final report, and utilizes between 100 and 150 animals. The purpose and principle of the test method are described in EPA's Health Effects Test Guidelines OPPTS 870.3700. The test substance is administered to pregnant animals at least from implantation to one day prior to the expected day of parturition. For inhalation developmental toxicity studies in rodents, the exposure regimen is usually 6 hr/day for gestation days 6 through 15. Shortly before the expected date of delivery, the pregnant females are terminated, the uterine contents are examined, and the fetuses are processed for visceral and skeletal evaluations. Because of the concern for chemicals that may produce malformations in humans but are not detected in rodent animal models (e.g., thalidomide), a non-rodent animal model using rabbits is often considered. The testing of rabbits for developmental toxicity is a separate study from that of rodents and would be an additional cost.

Reproductive Toxicity Study

Reproductive toxicity covers all phases of the reproductive cycle, and includes impairment of male or female reproductive function or capacity and the induction of nonheritable adverse effects on offspring (including death, growth retardation, structural abnormalities and functional effects). A two-generation reproduction study in rats typically costs $450,000, requires approximately twelve months from initiation to final report, and utilizes about 150 rats initially. The test substance is administered to parental animals prior to and during their mating, during the resultant pregnancies, and through the weaning of their F_1 offspring. The substance is then administered to selected F_1 offspring during their growth into adulthood, mating, and production of an F_2 generation, until the F_2 generation is weaned. Data from hundreds of pups are generated from the two generations of reproduction. The purpose and principle of the test method are described in EPA's Health Effects Test Guidelines OPPTS 870.3800. Briefly, the two-generation reproductive toxicity study is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. Additionally, since the study design includes *in utero* as well as postnatal exposure, this study provides the opportunity to examine the susceptibility of the immature/neonatal animal. For further information on developmental effects and functional

deficiencies, additional study segments can be incorporated into the protocol, utilizing the guidelines for developmental toxicity (described previously) or developmental neurotoxicity.

Other Studies

The most definitive test for carcinogenic potential using laboratory animal models is a chronic (lifetime) exposure of the chemical at a maximum tolerated dose. For rodents, this is generally referred to as a two-year bioassay. For most chemicals, the subchronic (e.g., 90-day) toxicity study and the *in vitro/in vivo* genetic toxicity test battery are sufficient to assess the potential for long-term toxicity and carcinogenicity. Carcinogenicity studies are not generally conducted for nonregulated chemicals for which the exposures are primarily occupational. An inhalation carcinogenicity study in rodents typically costs $1-1.5 million, requires approximately 36 months from initiation to final report, and utilizes a minimum of 480 rats or mice. The purpose of this study type is described in EPA's Health Effects Test Guidelines OPPTS 870.4300. If a two-year bioassay is conducted, it is important to keep in mind the extreme conditions (i.e. lifetime exposure at the maximum tolerated dose) used to elicit a tumorigenic response when interpreting the results.

Neurotoxicity studies examine the potential functional and morphological effects of chemical exposure to the nervous system that may occur in young adults or, in the case of developmental neurotoxicity, the offspring from maternal exposure during pregnancy and lactation. Some neurotoxicity tests are designed to assess specific neurotoxic endpoints, such as acetylcholinesterase or neurotoxic esterase activities (EPA's Health Effects Test Guidelines OPPTS 870.6100). More recently, specific tests have been developed for assessing a chemical's potential to produce immunotoxicity and endocrine disruption. The neurotoxicity, developmental neurotoxicity, immunotoxicity, and endocrine disruptor tests may be considered if there is reason to suspect alterations in the nervous, immune, or endocrine systems.

Metabolism and toxicokinetic studies are useful for determining the uptake of a chemical by the body from various exposure routes, distribution of the chemical in the organs and tissues of the body, and elimination of the chemical and its metabolites from the body. Also, metabolism and toxicokinetic studies are useful for setting dose/exposure levels for subchronic (e.g., 90-day) and chronic (e.g., carcinogenicity) studies and for extrapolating toxicology data from animals to humans. A toxicokinetic study typically costs $50,000 to $200,000, requires approximately four to twelve months from initiation to final report, and utilizes a minimum of four to greater than

100 animals, depending on study design. Analytical methods are an important component of toxicokinetic studies. Although this report does not recommend specific levels of concern for results from toxicokinetic studies, issues such as a long half-life for a chemical or metabolism to a reactive chemical intermediate (e.g. epoxides, free radical, etc.) tend to raise the level of concern for a chemical.

SUMMARY OF TOXICITY TESTING PROGRAM

A strategy to human health safety evaluation of halon replacement candidates is provided. A step-wise approach in building a chemical toxicity database, specific for halon replacement candidates, allows decisions to be made with budget and time constraints in mind. Four phases of toxicity testing are described. The confidence in predicting human health hazard increases as one proceeds from one phase of testing to the next, but the cost of each phase increases as well. Information is provided at the end of each phase that allows one to evaluate the overall benefit and cost of the tests performed before deciding to continue or stop toxicity testing of the replacement candidate. Figures 1 and 2 are flow diagrams that outline the strategy to be taken for toxicity testing Phases 1 and 2. Table 11 summarizes the cost and time associated with each of the phases of testing that are described in detail in this report. Figure 3 summarizes the relationship between duration, cost, gain in confidence, and likely regulatory decision for each of the four phases of toxicity testing.

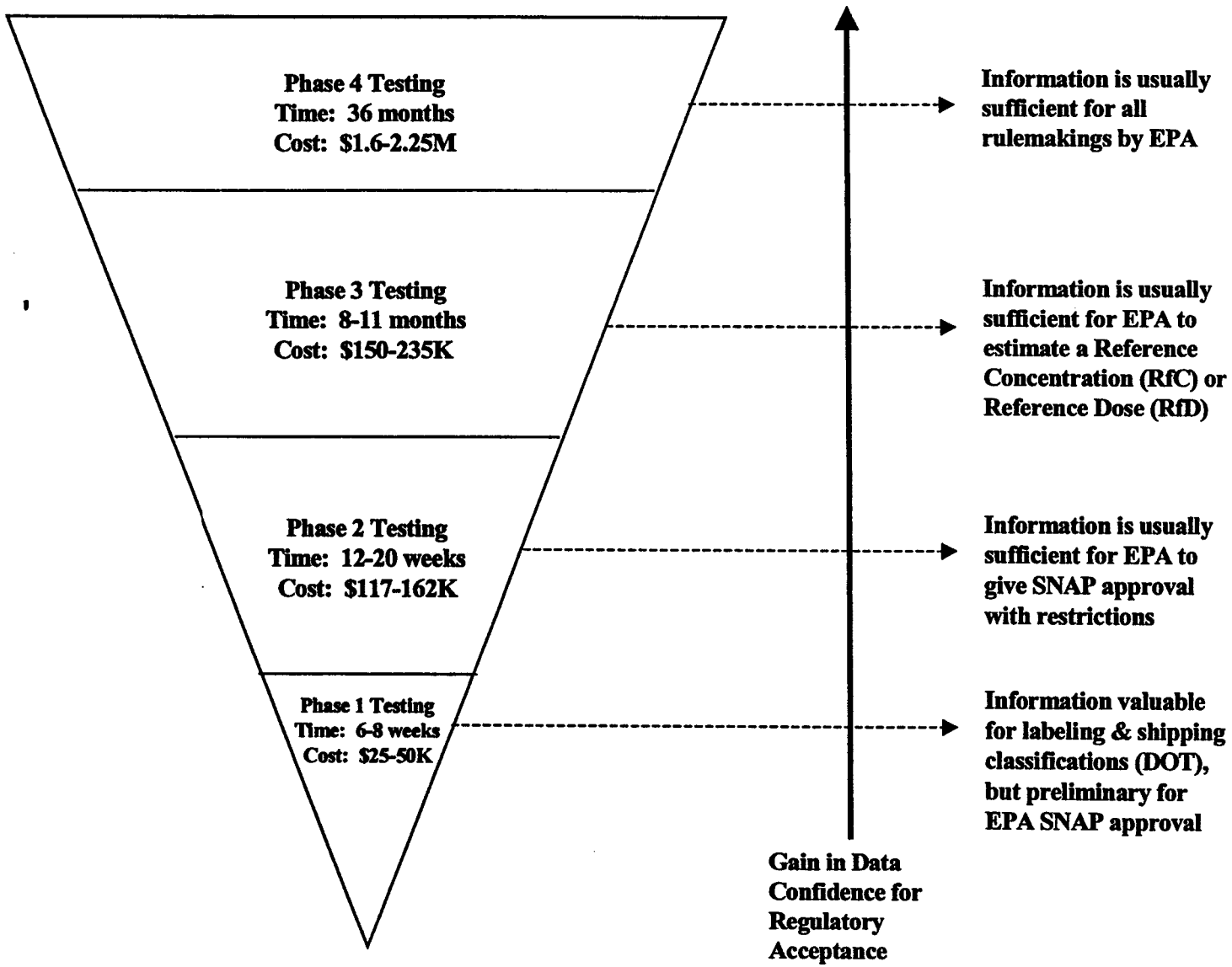
Table 11. Summary of Toxicity Testing of Halon Replacement Candidates

Phase	Test or Method	Estimated Cost ($)*	Time for Completion (start to final report)
1	Chemical/physical properties; literature search; define specific use scenario; SARs	10,000**	7 days**
1	*In vitro* screening	1,500 – 3,500	2 weeks
1	Acute irritation	1,500 – 2,000	3 weeks
1	Acute toxicity and "specific use" scenario test	7,500 – 15,000	4 weeks
1	Genotoxicity (*in vitro* bacterial cells)	7,000 – 17,000	6 – 8 weeks
2	Cardiac sensitization	40,000 – 65,000	6 – 8 weeks
2	PBPK modeling link to cardiac sensitization	10,000 – 25,000	4 – 12 weeks
2	14-Day dose range-finder	50,000	6 weeks
2	Genotoxicity (*in vitro* mammalian cells)	17,000 – 22,000 (higher for inhalation)	2 – 3 months
3	90-Day subchronic study	130,000 – 200,000	6 – 8 months
3	Genotoxicity (*in vivo* mammalian)	20,000 – 35,000	2 – 3 months
4	Developmental toxicity	100,000	8 months
4	Reproductive toxicity	450,000	12 months
4	Carcinogenic 2-year bioassay	1,000,000 – 1,500,000	36 months
4	Metabolism/toxicokinetics	50,000 – 200,000	4 – 12 months

*1999 Estimates

**Nyden and Skaggs, 1999

Figure 3. Relationship of Phase Testing with Regulatory Decision Making



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Appendix A

Protocol for the

Genotoxicity Screen of Volatile Compounds

HUMAN HEALTH SAFETY EVALUATION OF HALON REPLACEMENT CANDIDATES

Protocol for the Genotoxicity Screen of Volatile Compounds

I. Overall Objectives:

Research will be conducted to determine the potential genotoxicity associated with the exposure to (test material), a chemical that is being considered as a fire suppressant for potential military application.

A short-term genotoxic assay, Salmonella/Mammalian microsome reverse mutation assay, per EPA (OPPTS) Health Effect Testing Guidelines (870.5100, 1998), will be used to examine the genotoxicity of this volatile chemical.

II. Salmonella/Mammalian Microsome Reverse Mutation Assay

II-A. Purpose

The Salmonella/Mammalian microsome reverse mutation system is a microbial assay which measures the reversion from his- (histidine dependent) to his+ (histidine independent) induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.

II-B. Background

A reverse mutation assay using *Salmonella typhimurium* detects mutations in a gene of a histidine requiring strains to produce a histidine independent strain of this organism. A reverse mutation can be achieved by base pair changes, which may occur at the site of the original mutation or at a second site in the chromosome; or by frameshift mutations resulted from the addition or deletion of single or multiple base pairs in the DNA molecule.

In the standard plate incorporation *Salmonella*/mammalian microsome reverse mutation assay, the test agent, bacteria and the metabolic activation mixture (+S9) or buffer (-S9) will be mixed with 45^0 C overlay agar, spread on a plate of bottom agar and incubated for 48 hours at 37^0 C. However, volatile chemicals may evaporate before there is adequate time for bacteria to be exposed to the chemical. Therefore, in this assay, a vaporization technique will be used for test materials which are volatile at ambient temperatures. Briefly, the bacteria which are in the top agar with and without a metabolic activation system and plated onto histidine deficient minimum agar medium will be exposed to the vapor phase of a test agent in a Tedlar bag which will increase the contact time between the volatiles and the bacterial test system. After a suitable period of exposure, plates will be removed from the bag to avoid extra toxicity and contamination, and incubate for an additional 24 to 48 hours. Revertant colonies will be counted and compared with the number of spontaneous revertants in an untreated (air) and / or vehicle control culture. The mutagenicity of the test agents will be evident from the increased number of revertants.

II-C. Test Methods

1. Tester strains:

Two most sensitive tester strains for volatile chemicals with boiling points below 63 °C, will be used in this assay, which included TA100 for the detection of GC base pair mutagens, and TA102 for the detection of AT base pair mutagens (Hughes et al, 1987). The tester strains will be obtained from Dr. Bruce N. Ames in University of California, CA.

2. Confirmation of the genotypes of the tester strains:

Following genotypes will be confirmed in each tester strain based on the methods described by Maron and Ames (1983) prior to the mutagenesis study:

a. Requirement of histidine for growth (His^-)
b. Sensitivity to Crystal violet (rfa mutation)
c. Resistance to ampicillin or ampicillin +tetracycline for TA102 only (R factor)
d. Sensitivity to U.V. light (uvrB factor).
e. Spontaneous revertants

3. Bacteria growth:

Fresh culture of the tester strains will be used for each assay. The bacteria are cultured in nutrient broth at 37°C in a environmental shaker incubator for 10-12 hours (no more than 16 hours) to reach the late exponential or early stationary phase of growth (10^8-10^9 cells per ml).

4. Metabolic activation:

The test compound will be examined both in the presence and absence of an appropriate metabolic activation system. The most commonly used activation system in this assay is S9 mixture, a cofactor supplemented postmitochondrial fraction prepared from the liver of rats treated with enzyme inducers such as Aroclor-1254.

5. Test agent preparation:

The vapor phase test atmospheres of volatile chemicals will be prepared fresh in Tedlar® bags as they are needed. A measured volume of sterile breathing quality air is injected into an evacuated Tedlar® bag. The volume of air injected should not exceed 75% of the volume of the bag. A measured volume of the volatile liquid chemical will then be injected into the inflated bag. The bag will then be warmed (Pegram 1997) to ensure complete vaporization of the liquid chemical. Mixing of the chemical vapor and air will be accomplished by alternately pressing and releasing the bag several times. After preparation of the test atmosphere the bag should be stored in the dark or under yellow lights (Pegram 1997) and allowed to return to room temperature. As the bag returns to room temperature the chemical vapor may condense on the inside surface of the Tedlar® bag. This indicates that a saturated vapor is present in the bag and is a condition that should be avoided because of concentration stability problems. The chemical atmosphere contained in this bag will be the standard used to prepare other test atmosphere concentrations.

Each test atmosphere concentration will require a separate Tedlar® bag. For each desired vapor concentration, a measured volume of the test atmosphere prepared above is injected into the bag. Then, a measured of volume of sterile breathing quality air is injected. The contents of the bag is thoroughly mixed by alternately pressing and releasing the bag several times. The vapor concentration of each bag (determined by vapor phase dilution) will then be in a fixed ratio to the concentration in the standard bag which can be measured or calculated. A separate Tedlar bag will be used for the negative (air) control. For the dose selection a prescreening test using four test vapor concentrations will be conducted using both strains. Toxicity will be evident by a reduction in the spontaneous revertants per plate, and /or a clearing of the background lawn. Five test atmosphere concentrations ranging from low to high with adequate intervals will be selected and tested in the mutagenesis assay.

6. Controls:

In each assay, following concurrent controls will be set up:

a. Negative (air) controls

Cultures with and without S9 mixture will be exposed to sterile air as described in the chemical preparation except no chemical will be injected into Tedlar bag. They are used for the measurement of spontaneous revertants, which will serve as the background level of reverse mutation. Appropriate solvent controls will also be included in each assay by using regular plate incorporation method.

b. Positive control

Positive controls with known mutagens shall ensure the responsiveness of the tester strains as well as the efficacy of the activation system. Anthramine will be used as positive control with activation system (+S9) for both tester strains. In the non activation system (-S9), the positive controls will be sodium azide for TA 100 and Mitomycin C for TA102 . The above positive control agents will be dissolved in DMSO and tested using the regular plate incorporation method.

7. Mutagenesis assay

A Tedlar ®bag vaporization technique will be used in this assay. All dose groups (with and without S9 mixture) will be set up in triplicates. 0.1 ml of the culture is added to 2 ml of top agar which is melt and held at 45°C heating block along with 0.5 ml of S9 mixture or incubation buffer The contents are mixed and poured onto the surface of a minimum glucose agar plate. After the top agar solidifies, the plates with the lid on the bottom are placed in a Tedlar® bag. The bag is then sealed and evacuated. The contents of the Tedlar bag (prepared in Step 5) containing the desired chemical vapor concentration are transferred into the evacuated bag (do not fill the bag with the plates to more than 50% of it's capacity). The bag with the plates and chemical vapor will then be incubated at 37°C for 24 hours. After 24 hours of exposure, plates will be removed from the bag and incubated for an additional 24 hours for TA100 and 48 hours for TA102. Negative (air) control will be included in each experiment. Regular plate incorporation method will be used for solvent and positive control. The number of revertants per dish will be counted manually or by an automatic colony counter.

II-D. Data collection and reporting

The number of revertants per dish will be determined and the results are stored and processed by a computer using Excel spreadsheets. Following specific information will be reported: (1) Tester strains used (results of genotypic confirmation), (2) Metabolic activation system used (source, amount, cofactors, method for preparation), (3) Test agent preparation, dose levels and the rationale for their selection, (4) Positive and negative controls, (5) Individual plate counts, means, and standard deviation, and (6) Dose response relationship if applicable.

II-E. Result analysis and interpretation

1. Criteria for acceptability:

The data generated will be considered acceptable if:

a. The spontaneous revertant frequency is in the normal range as reported in the literature or within the laboratory's historical range.

b. A sufficient number of nontoxic concentrations have been tested.

c. The strain-specific positive mutagens significantly increase the revertant in the corresponding strains.

2. Criteria for interpretation:

a. Positive result:

A compound will be considered positive in this assay if a dose-dependent increase in the number of revertants is observed in three concentrations, or the highest increase equal to two times the spontaneous control value (Brusick, 1994). Sometimes the precise fold increase will not be necessary if a clear dose-dependent pattern is noted over several concentrations. A positive result in Salmonella/microsome mutagenesis indicates that under the experimental conditions, the test compound induces point mutation by base changes or frameshift in the genome of this organism.

b. Negative result:

A test agent will be considered negative in this assay if the criteria for positive response are not met, and the tester strains are sensitive to the positive mutagens.

A negative result indicates that under the experimental conditions, the test compound is not mutagenic in Salmonella typhimuriumm

III. Identification, Handling and Storage and of the Test Agent

The test agent will be provided in liquid form by the sponsor. All chemical and physical identifications will be verified and the sponsor will provide information. The compound is presumed to be soluble in DMSO and stable at ambient temperature. It will be kept in original container and stored at appropriate temperature, avoiding direct sunlight and sudden temperature rise. Safety glasses, rubber gloves, and protective clothing will be used for handling.

IV. Good Laboratory Practice and Quality Assurance

All assays will be conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Acts (EPA/TSCA) Good Laboratory Practice (GLP) Standards as defined in the Federal Register (40 CFR, Part 792, 1998) and the TSCA Test Guidelines (40 CFR 798.5265,1998). All the procedures are performed in accordance with the Standard Operating Procedures (SOPs) of ManTech Environmental for the Salmonella/microsome mutagenesis assay.

The Quality Assurance Auditor of ManTech Environmental Inc. will document inspections on all procedures used in this study. After the initiation of the study, modifications of the protocol will be in the form of Protocol Amendments, which will state the specific modifications and the reasons for the modifications.

V. Schedule

In the starting phase (about 4 weeks), all the test agents, media, equipments and tester bacteria will be ordered. The genotypes of the tester strains will be confirmed. The prescreening studies for dose selection will also be conducted in this period. The mutagenesis assay will be completed within 4 weeks including an independent confirmatory experiment. Another two weeks will be contributed to data analysis and report preparation.

VI. Reports and Deliverables

The technical part of the study is to be completed after 2 months of receiving the test material. A draft final report will be submitted after 2 weeks of completion of the study and the final report will be submitted one week after receiving review comments on draft final report from the sponsor. The final report constitutes the study's deliverable and ManTech considers acceptance of the deliverable to occur when it is received by U.S. Air force.

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